BRIEF REPORT: ABSENCE OF INTACT *nef* SEQUENCES IN A LONG-TERM SURVIVOR WITH NONPROGRESSIVE HIV-1 INFECTION

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ALTHOUGH disease develops within 10 years in most persons infected with human immunodeficiency virus type 1 (HIV-1), some remain symptom-free for prolonged periods.[1,2] Most long-term asymptomatic survivors of HIV-1 infection still have evidence of disease progression in the form of declining CD4+ lymphocyte concentrations. However, some rare persons not only are asymptomatic but also maintain stable levels of CD4+ lymphocytes in the normal or near-normal range. Although the definition of nonprogression may vary, approximately 5 percent of seropositive persons have shown no HIV-related disease or declines in CD4+ cell counts despite 10 or more years of documented HIV-1 infection.[2] Studying persons with long-term nonprogressive infection may help us to understand the mechanisms by which HIV-1 can be controlled.

Viral factors, host factors, or both may account for the absence of progression, at least in some persons. Host factors may include the inherent susceptibility of a person's cells to HIV-1 replication[3] or an HLA-determined ability to mount an adequate immune response.[4,5] Since most HIV-1 infections appear to result from only one or a few infectious viral particles,[6,7] we reasoned that a partially defective or attenuated strain of HIV-1 may be all that is transmitted in some cases. We focused our initial studies on the HIV-1 auxiliary gene called *nef*. This gene is not required for viral replication in cell culture, but *nef* is required in simian immunodeficiency virus (SIV) for the development of acquired immunodeficiency syndrome (AIDS) in rhesus monkeys.[8] The function of *nef* has not been clearly defined.

We amplified HIV-1 *nef* sequences from five patients with long-term nonprogressive HIV-1 infection. In one patient all 34 positive reactions from blood samples obtained over a decade yielded only defective forms of *nef*. The clinical and virologic characteristics of the HIV-1 infection in this man are strikingly similar to the characteristics of infection in rhesus monkeys with a strain of SIV missing *nef*. These results indicate that infection with attenuated forms of HIV-1 contributes to the absence of disease progression in some persons. They also provide further justification for considering the use of HIV-1 mutants with deletions as live attenuated vaccines.

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METHODS

DNA Analysis

We amplified HIV-1 DNA sequences spanning *nef* with the polymerase chain reaction (PCR), using nested primers as described elsewhere,[9] except that HIV-1–specific primers were used. The oligonucleotides used as primers were selected on the basis of their high level of sequence homology with most HIV-1 isolates in the Los Alamos data base.[10] The first round of amplification involved primers corresponding to nucleotides 8675 through 8698 and 9530 through 9507 of NL43[10] (5′GCAGTAGCTGAGGGGACAGATAGG3′ and 5′CCAGTACAGGCAAAAAGCAGCTGC3′). For the second round of amplification, 5 μl of the 100-μl reaction mixture was used with primers corresponding to nucleotides 8748 through 8762 and 9451 through 9439 (5′GCAGAGAATTCGAAGAATAAGACAGG3′ and 5′CCAGGCGAATTCTCCCTGGAAAGTCCC3′).

The sequences presented in this report have been submitted to GenBank (accession numbers U17438 to U17472).

Patients

Blood samples were obtained from patients followed by the New England Area Comprehensive Hemophilia Center at the Medical Center of Central Massachusetts, Memorial Hospital, Worcester. This cohort has been monitored since 1983 as part of a prospective study of immunoregulatory defects in hemophilia.[11] All participants have given informed consent. Most are seropositive for HIV-1 and were infected before 1983 through infusions with contaminated factor VIII concentrates. Lymphocyte subgroups were counted during 1991 and 1992 to determine whether there was disease progression in patients followed since 1984 or earlier. Patients were considered to have nonprogressive HIV-1 infection if they remained asymptomatic without ever having received antiretroviral therapy and if they had a CD4+ lymphocyte count of more than 400 per cubic millimeter and more than 30 percent of total T cells or a count of more than 600 per cubic millimeter irrespective of the percentage of CD4+ lymphocytes. Seven patients met these criteria. Patients were considered to have progressive infection if they had a CD4+ lymphocyte count of less than 200 per cubic millimeter and less than 20 percent of total T cells or of less than 100 per cubic millimeter irrespective of the percentage of CD4+ lymphocytes. Seventy-two patients met these criteria; patients who had died of HIV-related causes were also included in this category. Forty patients who did not meet the criteria for either of these categories were considered to have slowly progressive infection.

CASE REPORT

Patient 1 is a 14-year-old man with severe hemophilia A. He was exposed to HIV-1 through infusion of contaminated factor VIII concentrates before 1983 and has been consistently HIV-1–positive on Western blotting since first being tested in 1983. He has used recombinant factor VIII (Kogenate, Miles, West Haven, Conn.) twice a week since August 1988. The most recent physical examination was notable only for hemophilic arthropathy. Serologic tests are positive for hepatitis B surface antibody, cytomegalovirus antibodies, and hepatitis C antibodies. Aspartate aminotransferase and alanine aminotransferase levels have been elevated intermittently, but other chemical and hematologic laboratory values have remained unremarkable. Lymphocyte surface markers have been evaluated annually. CD4+ lymphocyte counts have remained stable and are only

slightly below the mean values in the HIV-1–seronegative members of this group of patients with hemophilia (Fig. 1).

Results

DNA was prepared from peripheral-blood mononuclear cells (PBMC) obtained in 1993 from five of the patients with nonprogressive HIV-1 infection, and sequences spanning the *nef* gene were amplified by PCR. Full-length *nef* sequences overwhelmingly predominated in four of the five patients. All positive PCR amplifications (33 of 33) from these four patients yielded a full-length fragment corresponding to the wild-type strain by gel analysis, and 23 of 26 DNA sequences from these 33 positive reactions had an intact *nef* open reading frame; 3 had deletions of a single base pair (bp) (data not shown). Others have previously shown that full-length *nef* genes predominate in HIV-1–infected persons with evidence of disease progression.[12,13] In contrast, only forms of *nef* with deletions were detected in PBMC obtained from Patient 1 in 1993 (Fig. 2 and 3).

Frozen PBMC that had been obtained from Patient 1 in 1983, 1986, and 1989 were used to prepare additional DNA samples for PCR amplification; 78 PCR amplifications were performed with PBMC obtained in 1983, 1986, 1989, and 1993. Despite the use of a sensitive, nested PCR procedure,[9] only 34 of 78 PCR amplifications yielded viral DNA when 2.5 μg of PBMC DNA was used per reaction tube. This result suggests that Patient 1 has a very low viral DNA burden. Measurement of HIV-1 *gag* DNA in PBMC by a different quantitative method[14] indicated the presence of approximately 1 viral DNA copy per 100,000 PBMC. This level was 10 to 3500 times lower than the level in PBMC from five of six patients with progressive or slowly progressive HIV-1 infection, which were evaluated by the same procedure around the same time. All 34 of the positive PCR results from Patient 1 yielded fragments shorter than that of full-length *nef* (Fig. 2).

DNA sequences were derived from clones obtained from the 34 positive PCR amplifications (Fig. 3). A single DNA fragment was observed by gel analysis in 33 of the positive reactions, and a single clone from each of these was used for sequencing. One of the reactions with the sample obtained in 1986 yielded two fragments (Fig. 2), and single clones representing each of the two sizes were used for sequencing (86F1 and 86F2 in Fig. 3). A variety of deletions were observed among the different clones (Fig. 3). The predominant deletion was 118 bp in length and was located in the *nef*-unique portion from nucleotides 8887 to 9004. This deletion removes a highly conserved acidic domain and a highly conserved $(Pxx)_4$ motif and places downstream sequences out of frame. Although a number of deletions were present in the region of *nef* that overlaps U3 in the long terminal repeat, none of the deletions affected *cis*-acting sequences known to be critical for viral replication — specifically, the polypurine tract, U3 terminal

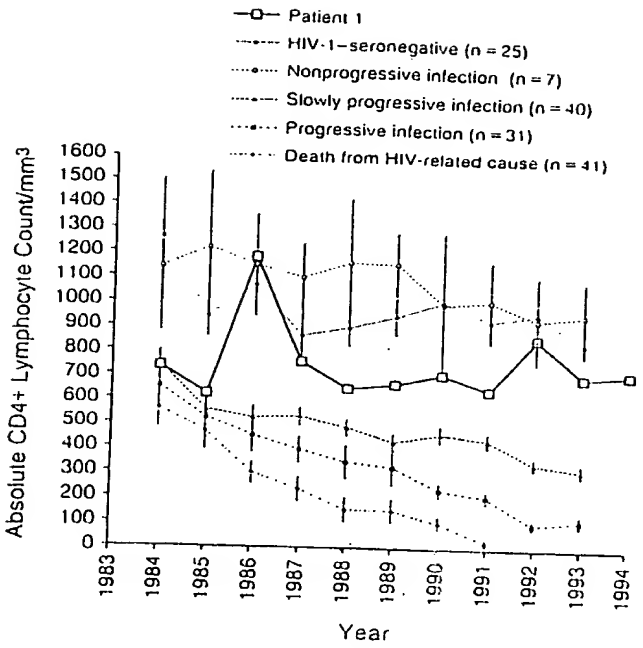

Figure 1. Mean (±SE) CD4+ Lymphocyte Counts in a Cohort of Patients with Hemophilia According to Their HIV-1 Status and Rate of Disease Progression.
The CD4+ lymphocyte counts for Patient 1 are shown separately.

sequences, TATAA box, and NFκB and Sp-1 binding sites (Fig. 3). Long deletions in U3 seemed to accumulate over time in Patient 1 only after 1983 (Fig. 3).

Attempts to recover HIV-1 from blood samples from Patient 1 have been repeatedly unsuccessful. Isolation attempts have included the use of serial dilutions of PBMC, whole blood, and plasma and bulk cultures of 5 million to 10 million PBMC cultivated with phytohemagglutinin-stimulated donor PBMC. Antigen-capture assays for p24 in plasma with immune-complex dissociation (Coulter Immunology, Hialeah, Fla.) have all been negative. Serum samples obtained in 1985 and plasma samples obtained in 1994 were negative for antibodies to HIV-1 nef protein by Western blotting at all dilutions tested (1:100 to 1:100,000); a pool of HIV-1–positive serum was positive for antibodies to nef protein in the same assay to a dilution of 1:10,000. Serum from five other patients with progressive HIV-1 infection was also examined, and all five were found to have antibodies reactive to nef. *Gag*-specific cytotoxic T lymphocytes, measured by limiting dilution assays as described previously,[5] were present at frequencies of 69 to 166 per million PBMC in freshly isolated, unstimulated samples obtained from Patient 1 at three points between November 1991 and March 1994. Env (IIIB)–specific cytotoxic T lymphocytes have also been demonstrated at similar levels. Cytotoxic T-lymphocyte precursors specific for nef were not demonstrable in an assay in

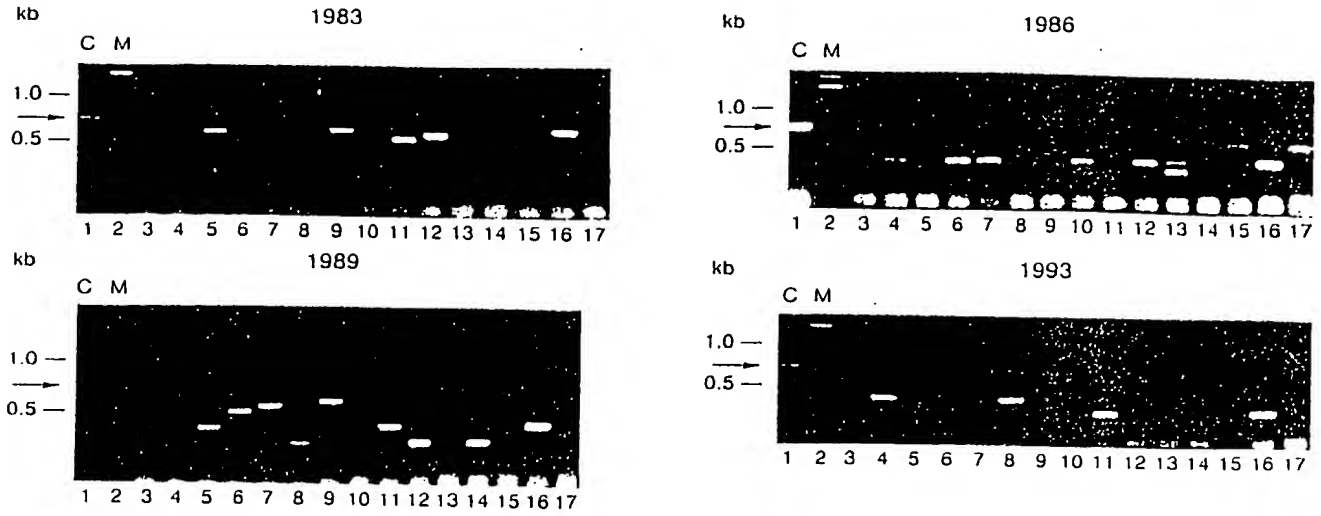

Figure 2. Analysis of *nef* Sequences in PBMC Obtained from Patient 1 in 1983, 1986, 1989, and 1993.
DNA fragments derived by PCR were separated by electrophoresis through 1.5 percent agarose gels. Lane 1 shows the PCR product of PBMC DNA from another patient with long-term nonprogressive HIV-1 infection, which was used as a control (C); lane 2 shows the size marker (M), a 1-kb ladder (GIBCO BRL); and lanes 3 through 17 show the products of individual PCR amplifications of DNA from PBMC obtained from Patient 1. The arrows indicate the position of the full-sized *nef* fragment.

which *gag* and *env* cytotoxic T-lymphocyte precursors were measured at frequencies of approximately 300 per million PBMC with nonspecific stimulation in vitro.[3] The persistence of antibodies and cytotoxic T-lymphocyte activity and the accumulation of additional deletions with time in the region of *nef* that overlaps U3 argue for the continued presence of replication-competent HIV-1, albeit at very low levels, in Patient 1.

Discussion

Rhesus monkeys inoculated with a derivative of the pathogenic SIVmac239 clone containing a 182-bp deletion in *nef* became infected and persistently antibody-positive. However, they had extremely low viral burdens, normal CD4+ lymphocyte concentrations, and no signs of disease progression.[8,9,13] These characteristics are strikingly similar to the course of infection in Patient 1. In these monkeys, additional deletions accumulate over time in the region of *nef* that overlaps U3, without affecting the critical *cis*-acting sequences.[9] This is also remarkably similar to the pattern described for Patient 1 and illustrated in Figure 3. The *nef* sequences that overlap U3 are apparently not advantageous to the virus in the absence of an intact *nef* gene and are selectively lost.

Most HIV-1–positive persons with hemophilia became infected in the early 1980s, before the advent of effective blood screening. Since Patient 1 was HIV-1–positive when first tested in 1983, we cannot be certain that he was infected initially with only *nef*-defective HIV-1. However, the marked disadvantage of SIV and HIV variants with *nef* mutations in vivo in animal models strongly suggests that this is the case. Strains of SIV with mutated forms of *nef* are strongly selected against in infected monkeys.[8] In addition, HIV-1 variants with mutations in *nef* are at a disadvantage as compared with the wild-type strain in mice with severe combined immunodeficiency that have been given human lymphoid cells[16] and in experimentally infected chimpanzees (unpublished data). At the very least, defective forms of *nef* with deletions have vastly predominated in Patient 1 since 1983, and he has had no disease progression. It is possible that there are additional defects elsewhere in the HIV-1 genome in this patient that could contribute to the attenuated phenotype. Our observations should stimulate additional investigations into the extent to which infection with partially defective viruses may correlate with the absence of disease progression.

In this report, we describe a particular HIV-1–gene defect associated with the absence of disease progression in a single patient. Our results, and those of Huang et al.,[17] suggest that deletions in *nef* may not be a common explanation for the absence of progression and that different factors are likely to contribute in other patients. Viral factors that could contribute include different types of mutations in a wide variety of viral genetic elements. Viral and host factors cannot be dissociated from each other, since an effective immune response is an essential feature of nonprogression. Disease outcome is likely to be determined by a delicate balance between the ability of the virus to replicate and the host's ability to mount an adequate immune response.

Rhesus monkeys infected with SIV with *nef* deletions are strongly protected against challenge with wild-type, pathogenic SIV,[13] and it has been suggested that

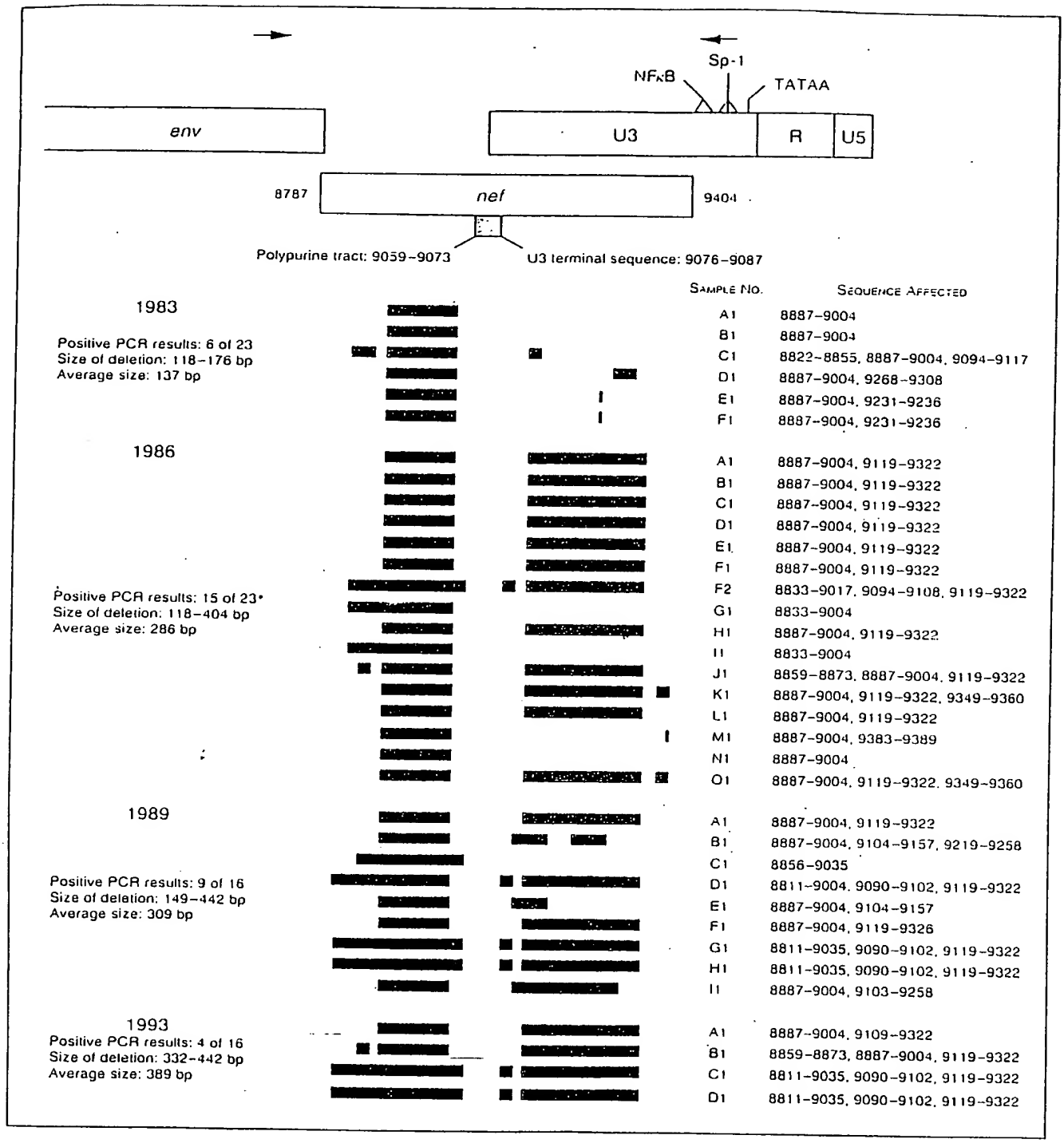

Figure 3. Location of *nef*-Sequence Deletions in PBMC Obtained from Patient 1 in 1983, 1986, 1989, and 1993.

The nucleotide numbers refer to those of the NL43 clone of HIV-1.[9] In the diagram the arrows indicate the locations of oligonucleotides used to amplify viral DNA. The asterisk indicates that one PCR produced two separate clones (F1 and F2) of different sizes.

derivatives of HIV-1 with multiple deletions should be considered for use as live attenuated vaccines.[15,18,19] Concern for safety is the key factor limiting further development of this promising approach. However, it should be remembered that disease progression is not an inevitable outcome of lentivirus infections. African green monkeys and sooty mangabey monkeys harboring their own SIV remain infected apparently for life, but do not seem to have active disease. Strains of SIV from these species are certainly capable of causing disease, because they do so in other hosts: Similarly, chimpanzees infected with wild-type HIV-1 and macaques infected with mutant strains of SIV remain infected but asymptomatic.[8,20,21] The immunologic responses of these species appear to be sufficient to control these lentiviral infections. By deleting portions of *nef* or other genetic elements, the balance of power may simply shift in favor of the host's immune response. The finding of only HIV-1 variants with *nef* deletions in a healthy man with long-term nonprogressive HIV-1 infection provides additional impetus for consideration of this vaccine approach.

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